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Suppressive Effects of Human Herpesvirus 6 on In Vitro Colony Formation of Hematopoietic Progenitor Cells

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Human herpesvirus 6 (HHV-6) has been reported to be involved in bone marrow failure after bone marrow transplantation (BMT). To elucidate the role of HHV-6 in the marrow failure, we examined the comparative effect of two variants of HHV-6 (HHV-6A and HHV-6B) and human herpesvirus 7 (HHV-7) on in vitro colony formation of hematopoietic progenitor cells in methylcellulose semi-solid media. Progenitor cells prepared from cord blood mononuclear cells (CBMNCs) were infected with one of these viruses at various multiplicity of infection (MOI), and were subjected to methylcellulose colony assay. Formation of both granulocyte/macrophage (CFU-GM) and erythroid (BFU-E) colonies was MOI-dependently suppressed after infection with the Z29 strain of HHV-6B. Although HHV-6A suppressed the formation of BFU-E colonies as efficiently as HHV-6B, the former did not exhibit significant suppressive effect on the formation of CFU-GM colonies at an MOI 1. HHV-7 had no effect on hematopoietic colony formation at all. Based on frequent positivity of viral DNA in single colonies obtained from HHV-6-infected progenitor cells by polymerase chain reaction and in situ hybridization, direct effects of HHV-6 on the hematopoietic progenitor cells are suggested as the cause of the suppression rather than indirect effects via accessory cells of the bone marrow. ***J. Med. Virol. 52:406–412, 1997.***

KEY WORDS: human herpesvirus 7; bone marrow transplantation; colony forming unit granulocyte/macrophage; burst forming unit erythroid; polymerase chain reaction; in situ hybridization

INTRODUCTION

Human herpesvirus 6 (HHV-6) was first isolated in 1986 from patients with lymphoproliferative disorders and acquired immunodeficiency syndrome [Salahuddin et al., 1986]. An etiological relation was clearly demonstrated by Yamanishi et al. [1988] between primary HHV-6 infection and exanthem subitum, a self limited childhood illness characterized by fever and rash. The virus is predominantly T lymphotropic [Lusso et al., 1991; Takahashi et al. 1989] and has been divided into two types, HHV-6A and HHV-6B, based on the variation of restriction fragment profiles, growth properties, and reactivity to monoclonal antibodies [Schimer et al., 1991; Ablashi et al., 1991]. Human herpesvirus 7 (HHV-7) is another T lymphotropic virus. It was isolated in 1990 from the activated T cells of a healthy individual [Frenkel et al., 1990]. As is the case with other herpesviruses, both HHV-6 and -7 are thought to cause latent infection after primary infection in childhood, and to reactivate either spontaneously or under immunosuppressive conditions. A variety of diseases, such as chronic fatigue syndrome, histiocytic lymphadenopathy, mononucleosis-like syndrome, and multiple sclerosis are reported to be associated with HHV-6 [Buchwald et al., 1992; Sumiyoshi et al., 1993; Akashi et al., 1993; Challonger et al., 1995].

Carrigan et al. [1994] reported that HHV-6 was isolated from 6 out of 15 bone marrow samples from adult patients who suffered clinically-diagnosed bone marrow suppression after allogeneic BMT. The virus isolation rate of these patients was higher than that of BMT patients without delayed engraftment. Five of these 6 isolates were identified as HHV-6B, while the other was a mixture of HHV-6B and -6A. Other reports [Yoshikawa et al., 1991; Wilborn et al., 1994; Wang et al. 1996] also suggested the involvement of HHV-6 in

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bone marrow suppression. On the other hand, a large scale study conducted recently [Kadakia et al., 1996] suggested that there is no significant difference in the rate of delayed engraftment between virus-isolation positive and negative patients, although active HHV-6 infections occur frequently after BMT.

We examined the comparative effect of two types of HHV-6 and HHV-7 on in vitro colony formation of hematopoietic progenitor cells prepared from cord blood [Nakahata et al., 1982] in methylcellulose semi-solid media.

MATERIALS AND METHODS

Preparation of Cord Blood Mononuclear Cells (CBMNCs)

After obtaining consent from maternal patients, cord blood was collected and heparinized. The samples were diluted with Hanks' balanced solution and were separated by the Ficoll-Conray density gradient method. Light-density cells (<1.077) were collected and washed twice in phosphate-buffered saline (PBS).

T-Cell Depletion From CBMNCs

T-cells were depleted from CBMNCs by rosette formation with neuraminidase-treated sheep red cells (SRBC) as previously described [Kaplan et al., 1974]. The T cell-depleted samples still contained approximately 5% T cells as determined by flow cytometry using anti-CD3 monoclonal antibody (Becton Dickinson, San Jose, CA).

Preparation of Viral Stocks

CBMNCs were cultured for 3 days in the complete medium, i.e., RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 5 ug/ml of phytohemagglutinin (PHA-P; Difco Laboratories, Detroit, MI) and 0.1 U/ml of recombinant interleukin-2 (IL-2; GIBCO BRL Life Technology Inc., Grand Island, NY). CBMNCs thus prepared were mixed with CBMNCs infected with either HHV-6 or HHV-7 at a ratio of 1:5 and cocultivated in the complete medium without PHA for 5–7 days. The Z29 strain of HHV-6B, the U1102 strain of HHV-6A, and the SB and MRK strains of HHV-7 were used. The supernatant of the infected culture containing high titer virus was clarified by centrifugation at 3000 rpm for 10 minutes, and finally stored at −80°C until use.

Titration of Viral Stocks

The 50% tissue culture infectious dose ($TCID_{50}$) of the virus stock was determined by infecting quadricate cultures of IL-2 and PHA activated CBMNCs with serial 10-fold dilution of virus stock as described previously [Yoshida et al.,1996].

Infection of T-Cell Depleted CBMNCs

T-cell depleted CBMNCs were resuspended in Iscove's modified Dulbecco's medium (IMDM) at a concentration of 1×10^6 cells/ml. The T-cell depleted CBMNCs were inoculated with 5 ml of each virus solution at various multiplicity of infection (MOI), centrifuged at 3000 rpm for 60 min, and washed twice with IMDM.

Methylcellulose Colony Assays

Infected cells were finally resuspended in semisolid matrix (METHLCULT GF H4434 StemCell Technology Ins, Vancouver, Canada) at a concentration of 5×10^4 cells/ml and were plated in triplicate into a six-well tissue culture plate (Lux Nunc). The semisolid matrix consisted of IMDM supplemented with 0.9% methylcellulose, 30% FCS, 1% bovine serum albumin, 3 U/ml erythropoietin, 10^{-4} M mercaptoethanol, 2 mM L-glutamine, 50 ng/ml recombinant stem cell factor, 10 ng/ml recombinant granulocyte/monocyte-colony stimulation factor, and 10 ng/ml recombinant interleukin-3. The plate was cultured in a 5% CO_2 humidified atmosphere at 37°C. Twelve days after plating, the number of CFU-GM (colony forming unit-granulocyte/macrophage) and BFU-E (burst-forming unit erythroid) colonies was counted under an inverted microscope. At the same time, the colonies were photographed.

Polymerase Chain Reaction (PCR)

Fourteen days after plating, each colony was individually aspirated with a micro-capillary pipette, suspended in PBS, and washed twice. DNA preparation was performed as described previously [Molina et al., 1990; Louache et al., 1992]. The HHV-6 sequence was amplified by PCR with the use of the primers described by Kondo et al. [1990]. The primers have shown to amplify both A and B types of HHV-6 and the product was 782 base pairs in length.

Cytospin Preparations of Colony-Forming cells

Each colony was individually aspirated by a micro-capillary pipette and suspended in PBS. The cell suspension was cytospun at 650 rpm for 5 minutes onto a 3-amino-propyl-triethoxysilane coated slide (Dako, Carpinteria, CA). The slide was air-dried, fixed with acetone, and stored at −80°C until use.

Immunofluorescence (IF) Staining

Two monoclonal antibodies specific for HHV-6, one directed to the gp106 glycoprotein (OHV1) [Okuno et al.,1992] and the other directed to the p41 early antigen (Universal Bio., Rockville, MD), were used as the first antibodies, and indirect IF staining was carried out as described previously [Yoshida et al.,1996].

In Situ Hybridization

Digoxigenin-labeled HHV-6 specific probes were generated by the PCR method [Saeki et al.,1993] using the primer described above. Each specimen was covered with 50 μl of hybridization solution containing 50% formamide, 10% dextran sulfate, 10 mM Tris-HCl (pH7.6), 1 mM EDTA, 600 mM NaCl, l × Denhardt's solution, 200 μg/ml *E. coli* tRNA, and the probe, and placed on a heating block at 95°C for 5 minutes. The

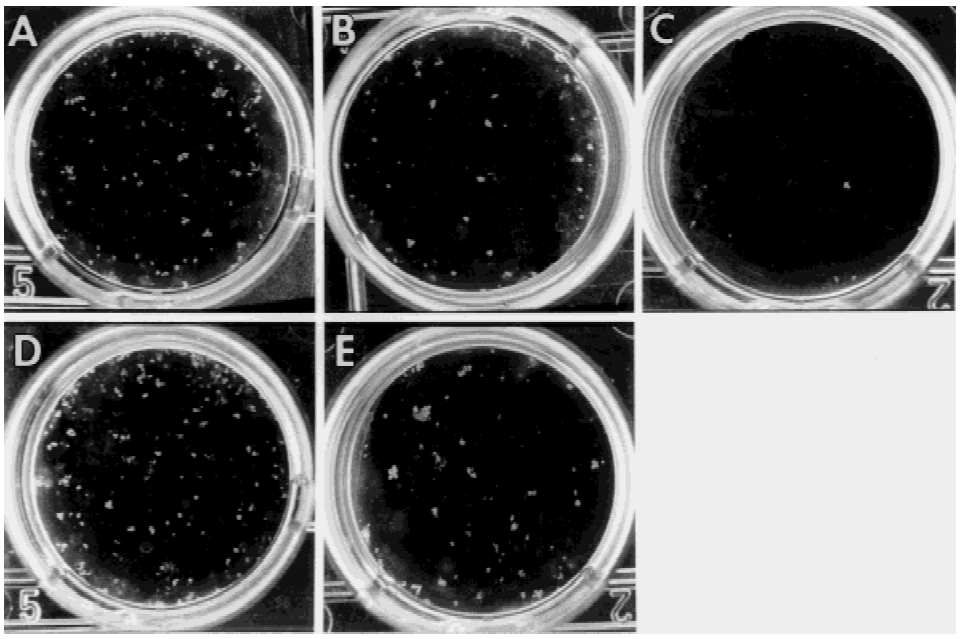

Fig. 1. CFU-GM and BFU-E colonies in semisolid culture 12 days after infection of CBMNCs. **A**: Mock-infected culture. **B**: Culture infected with HHV-6B (Z29 strain) at an MOI of 1 $TCID_{50}$/cell. **C**: Culture infected with HHV-6B (Z29) at an MOI of 10. **D**: Culture infected with HHV-7 (SB) at an MOI of 10. **E**: Culture infected with HHV-6A (U1102) at an MOI of 1.

slides were incubated in a moist chamber at 42°C for 16 hours. After a stringent wash, detection of the signal was performed using a DIG Nucleic Acid Detection Kit according to the manufacturer's recommendations (Boehringer Mannheim, Germany).

Statistical Analysis

Statistical analysis was carried out using a one way ANOVA followed by the Fisher's PLSD test.

RESULTS

The first experiment was conducted to compare the effect of HHV-6A, HHV-6B, and HHV-7 on colony formation of hematopoietic progenitor cells. T-cell depleted CBMNCs, used as a source of hematopoietic stem cells, were infected with either of these viruses, and maintained in methylcellulose-based matrix. As shown in Figures 1 and 2, HHV-6B inhibited both CFU-GM and BFU-E colony formation. HHV-6A suppressed BFU-E colony formation, while the inhibitory effect on CFU-GM colony was not significant. The colonies observed in the HHV-6B-infected cultures were smaller than those observed in mock-infected culture (Fig. 3). However, HHV-7 had no effect on colony formation at all (Figs. 1 and 2).

To confirm the effect of MOI with HHV-6B on the suppression of colony formation, five more experiments were undertaken using different sources of CBMNCs (Fig. 4). We observed a dose-dependent suppression of CFU-GM and BFU-E colonies in all the experiments.

The PCR was employed to detect the HHV-6 sequence from single colonies exposed to HHV-6A or HHV-6B (Fig. 5). The HHV-6 sequence was detected in single colonies of both CFU-GM and BFU-E origin. Table I shows the detection rates of the HHV-6 sequence in the colonies exposed to HHV-6B at various MOI. The HHV-6 sequence was more frequently detected in the colonies exposed at higher MOI.

To investigate further the mode of infection of HHV-6B in individual colony forming cells, in situ hybridization and immunofluorescence were carried out. Positive hybridization signals were detected in the nuclei of the cells forming CFU-GM colonies (Fig. 6). A similar result was obtained with BFU-E colony forming cells. However, very few viral antigen positive cells were detected in either CFU-GM or BFU-E colony forming cells by immunofluorescence using monoclonal antibodies against the p41 early antigen or the gp106 glycoprotein (Fig. 7).

DISCUSSION

It was demonstrated that HHV-6B inhibited both the CFU-GM and BFU-E colony formation in vitro in an MOI-dependent manner. The inhibitory effects of HHV-6A on colony formation were not as prominent as those of HHV-6B. High-titer viral stock of HHV-6A was not obtained, which would have permitted the infection at MOI of 5 or 10. Carigan and Knox [1995] reported potent suppressive effect of $HHV6_{GS}$, one of strains of variant A, even at a low MOI. There exist substantial differences in the experimental system, to which the discrepancy of the results might be attributable. They examined the colony formation of the bone marrow cells, while we examined the colony formation of T-cell-depleted CBMCs. They used virus stocks prepared from different T cell lines for A and B variants of HHV-

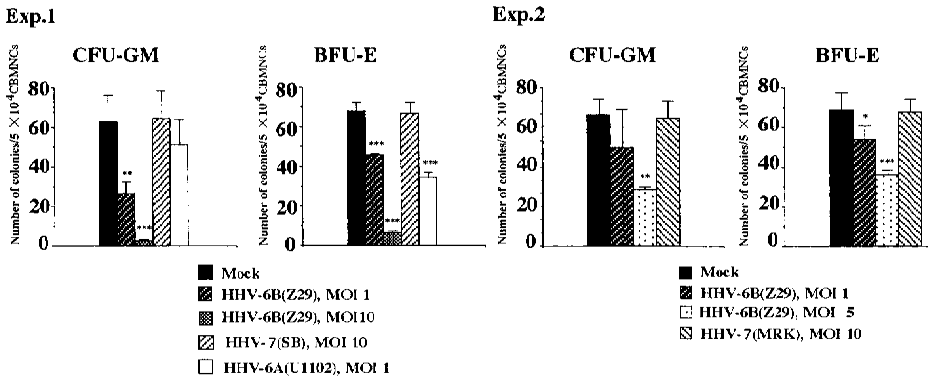

Fig. 2. Number of CFU-GM and BFU-E colonies in semisolid culture after infection of CBMNCs. The results are the means of triplicate cultures ± the standard error. The differences noted in this figure are significant when compared with the mock-infected culture; one asterisk (*) at $P < 0.05$, two asterisks (**) at $P < 0.01$, or three asterisks (***) at $P < 0.001$.

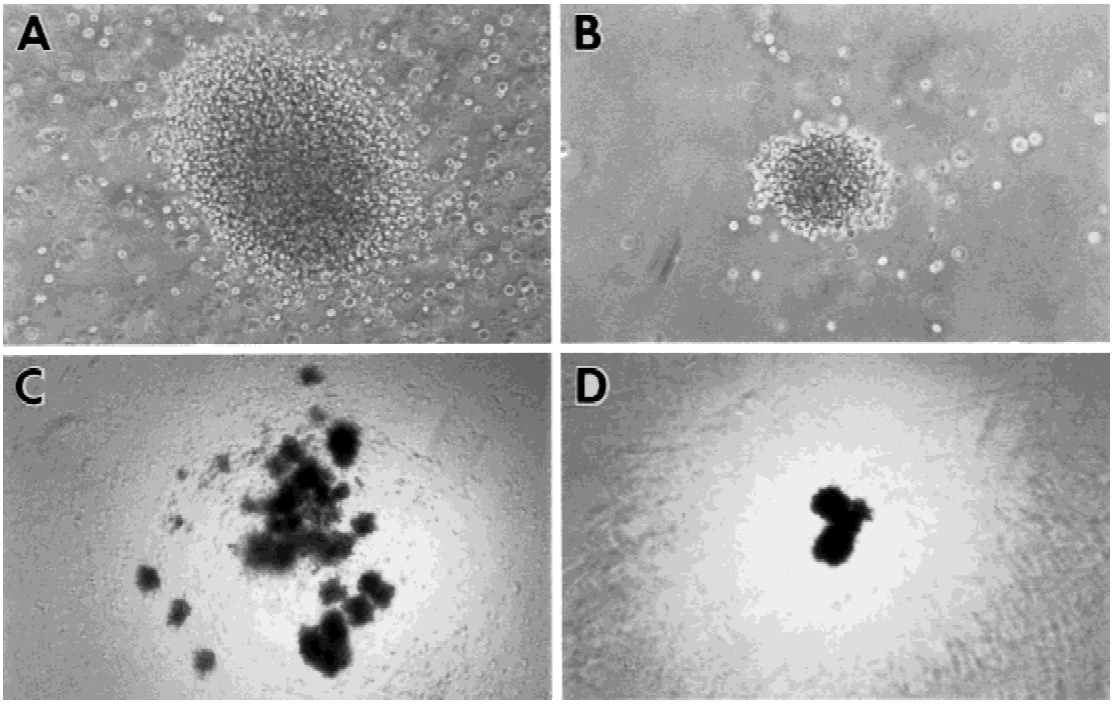

Fig. 3. CFU-GM and BFU-E colonies in semisolid culture 14 days after infection of CBMNCs as seen under an inverted light microscope. **A**: A CFU-GM colony in the mock-infected culture. **B**: A CFU-GM colony in the culture infected with HHV-6B (Z29 strain) at an MOI of 10 $TCID_{50}$/cell. **C**: A BFU-E colony in the mock-infected culture. **D**: A BFU-E colony in the culture infected with HHV-6B (Z29) at an MOI of 10. Magnification, × 60 for A and B, and × 30 for C and D.

6, while we prepared every virus stock from stimulated CBMCs. Moreover, difference between the GS and U1102 strains of HHV-6A is noted in biological properties.

HHV-7 did not have any suppressive effect on colony formation at all. There is no report to date on BM suppression after BMT associated with HHV-7. Differences in suppressive effects on colony formation between HHV-6 and HHV-7 might partly be attributed to the difference in the tropism between these viruses [Lusso et al.,1991]. Specifically, HHV-7 uses the CD4 molecule as a receptor for the entry to the host cells [Lusso et al., 1994; Yasukawa et al, 1995]. It is likely that the lack of effect of this virus on hematopoietic colony formation is due to the inability of the virus to infect hematopoietic progenitor cells. However, the susceptibility of these cells to HHV-7 should be examined precisely using purified $CD34^+$ cells.

Although the mechanism of BM suppression after BMT by HHV-6 is not clear, it is possible that reactivation of latent HHV-6 takes place under immunosuppressive conditions after BMT and that the amount of virus increases enough to have a suppressive effect on BM function. Two potential mechanisms have been suggested for the development of the suppressive effect by HHV-6. First, HHV-6 may directly infect hemato-

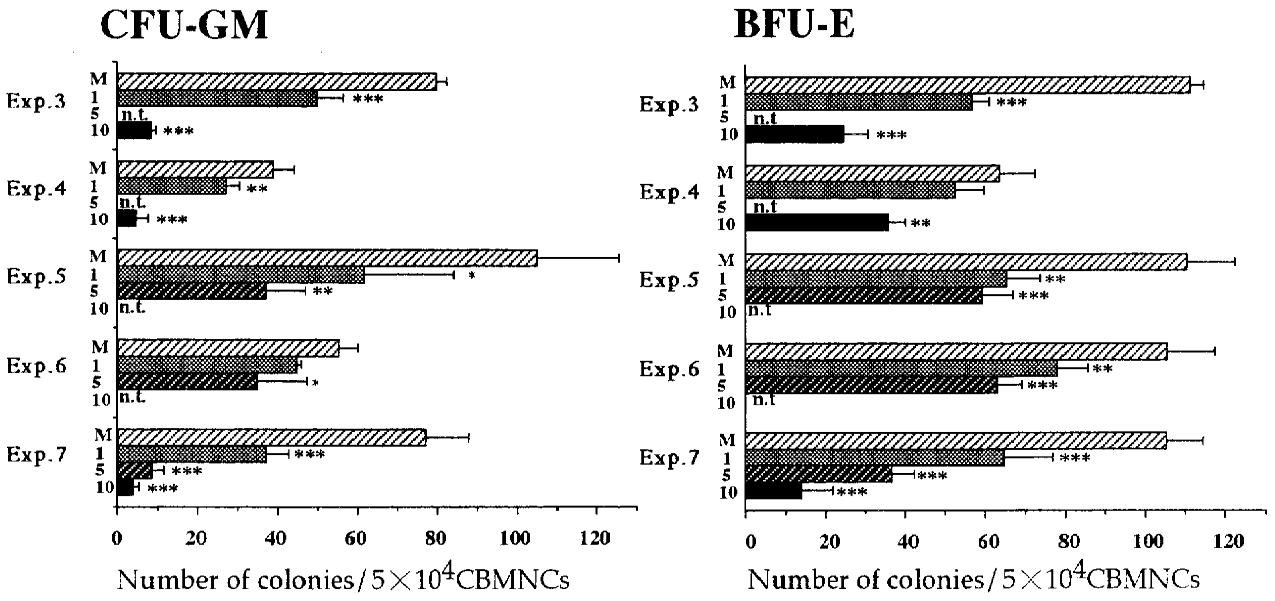

Fig. 4. MOI-dependent suppression of CFU-GM and BFU-E colonies in semisolid culture after infection of CBMNCs with HHV-6B (Z29). M; mock-infected, 1: at an MOI of 1 $TCID_{50}$/cell, 5: at an MOI of 5, 10: at an MOI of 10. n.t.; not tested. The results are the means of triplicate cultures ± the standard error. The differences noted in this figure are significant when compared with the mock-infected culture; one asterisk (*) at $P < 0.05$, two asterisks (**) at $P < 0.01$ or three asterisks (***) at $P < 0.001$.

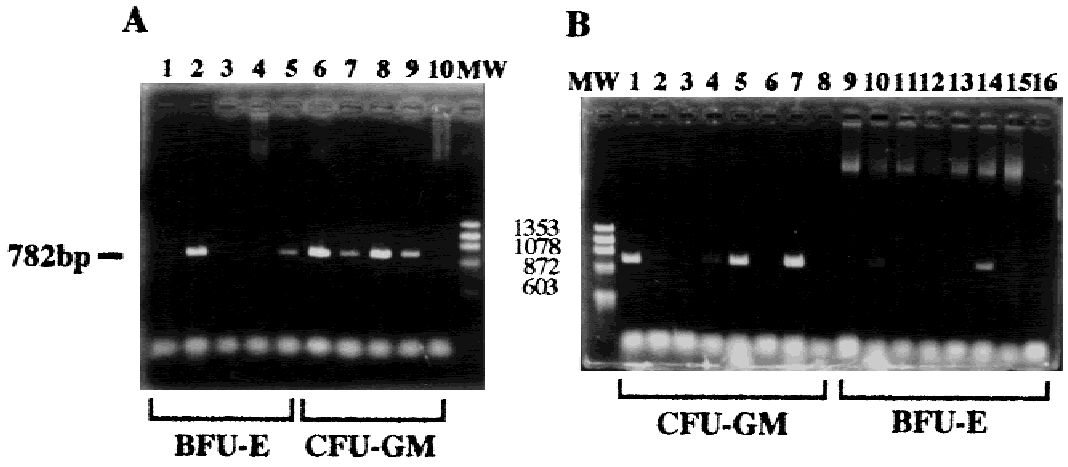

Fig. 5. PCR detection of the HHV-6 sequence from single colonies formed 14 days after infection of CBMNCs with HHV-6B(Z29) (**A**) at an MOI 10 or HHV-6A (U1102; **B**) at an MOI of 1. A, lanes 1 through 5: BFU-E colonies; lanes 6 to 10: GFU-GM colonies. B, lanes 1 through 8: CFU-GM colonies; lanes 9 to 16: BFU-E colonies. MW; molecular weight markers (ϕX174/Haelll digests).

poietic stem cells and/or committed hematopoietic progenitor cells and inhibit their growth. Alternatively, HHV-6 may infect bone marrow accessory cells and either inhibit the production of essential growth factors or induce the release of mitotic inhibitors.

The HHV-6 sequence was detected in single colonies of CFU-GM and BFU-E exposed to HHV-6 by PCR. In in situ hybridization virus DNA was detected in almost all colony forming cells of CFU-GM and BFU-E. Because all cells in each colony are believed to be derived from a single progenitor, it is not surprising that HHV-6 DNA was present in all daughter cells. Furthermore, a similar suppressive effect was observed on

TABLE I. Detection Rate of HHV-6 Genome by PCR in Single Colonies Formed After Infection of CBMNCs With HHV-6B (Z29) at Various MOI

MOI	Detection rate in	Exp. 3	Exp. 4	Exp. 5	Exp. 6
1	CFU-GM	1/5	0/8	1/5	3/12
	BFU-E	1/5	0/7	0/5	1/10
5	CFU-GM	n.t.	n.t.	3/6	5/8
	BFU-E	n.t.	n.t.	1/6	2/6
10	CFU-GM	5/5	4/5	n.t.	n.t.
	BFU-E	5/5	2/5	n.t.	n.t.

*Data are presented as the ratios of number of positive colonies/number of colonies tested. n.t., not tested.

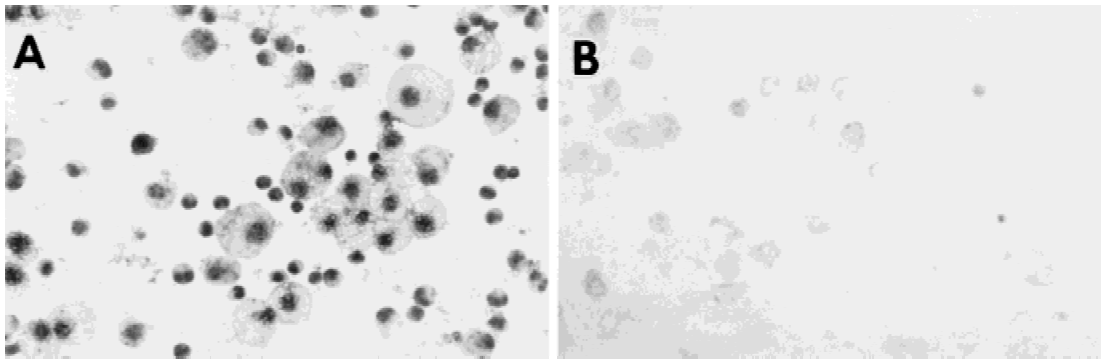

Fig. 6. Detection of HHV-6 genome with in situ hybridization. **A**: Cells obtained from a CFU-GM colony 14 days after infection of HHV-6B(Z29) at an MOI of 10. **B**: Cells obtained from a mock-infected CFU-GM colony as a negative control. Magnification × 100.

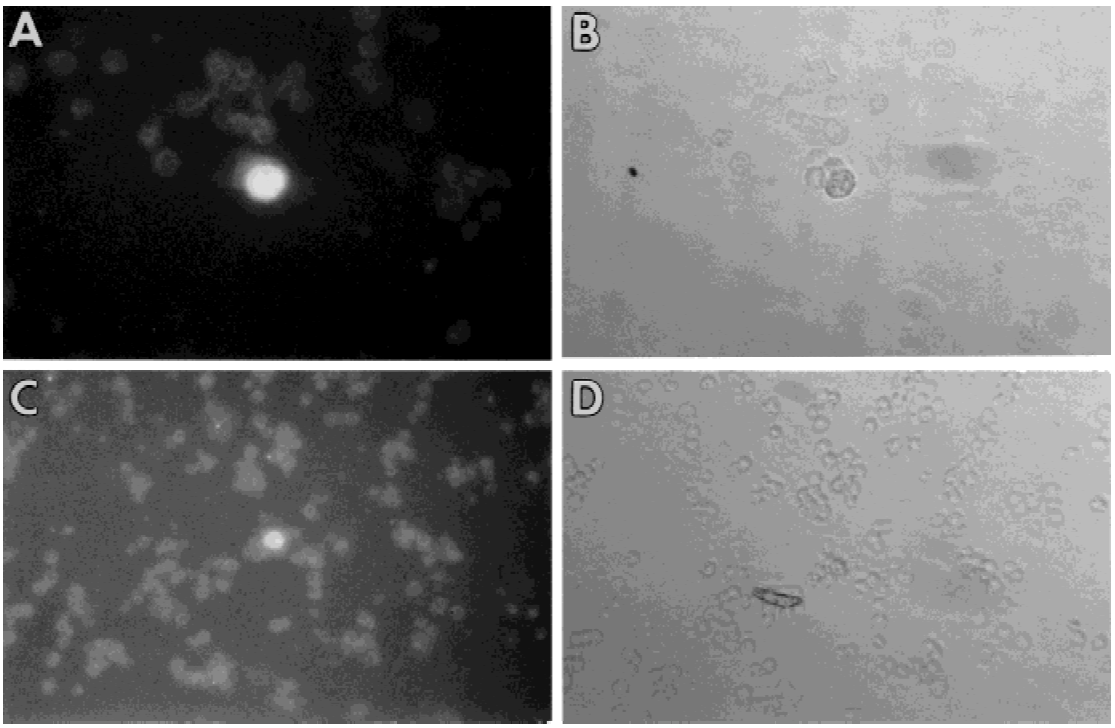

Fig. 7. Expression of a viral antigen in colony-forming cells 12 days after infection of HHV-6B(Z29) at an MOI of 10. Immunofluorescence (**A**) and phase contrast (**B**) images of the cells obtained from a CFU-GM colony. Immunofluorescence (**C**) and phase contrast (**D**) images of cells obtained from a BFU-E colony. A monoclonal antibody specific for HHV-6 gp106 (OHV1) was used as the first antibody of indirect immunofluorescence. Magnification × 100.

colony formation by purified $CD34^+$ cells exposed to HHV-6B (data not shown). These results support the former hypothesis that HHV-6 infects directly stem cells and/or committed hematopoietic progenitor cells and inhibits their growth. In hematopoietic progenitor cells which survived the initial infection, HHV-6 DNA was maintained in progeny throughout replication and differentiation, although the expression of viral antigens was very limited. Thus, two kinds of virus-host relationship are suggested; a lytic infection which is assumed to have occurred in suppressed colonies, and a latent/persistent infection in formed colonies with very little chance of the reactivation of virus production in a fraction of cells.

Related to this, Kondo et al. [1991] have shown that HHV-6 may infect latently monocytes in vivo and in vitro and that certain factors may reactivate it. It is also possible that hematopoietic progenitor cells might become infected with HHV-6 at primary exposure in infancy, and either remain in the bone marrow as a virus reservoir or generate infected progeny capable of migrating into the peripheral circulation. Thus we postulate that HHV-6-infected BM cells contribute to the dissemination or reactivation of the virus in immunosuppressed patients.

In conclusion, the experimental system in the present study demonstrated quantitatively the effects of virus on colony formation of hematopietic progenitor

cells and the results were highly reproducible even using different sources of CBMNCs. Using this system, it is planned to evaluate the suitability of various antiviral agents for the treatment of HHV-6-related bone marrow suppression.

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